

## Research report

## Oral uridine pro-drug PN401 decreases neurodegeneration, behavioral impairment, weight loss and mortality in the 3-nitropropionic acid mitochondrial toxin model of Huntington's disease

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## Abstract

Huntington's disease (HD) is associated with decreased activity of mitochondrial succinate dehydrogenase (complex II). De novo biosynthesis of uridine nucleotides is directly coupled to the respiratory chain. Cells with impaired mitochondrial function become uridine auxotrophs and can be maintained with high micromolar concentration of uridine and pyruvate. The therapeutic role of pyrimidines and possible changes in uridine content has not been assessed in neurological diseases involving mitochondrial dysfunction in vivo. Oral administration of PN401 delivers much higher levels of uridine to the circulation than oral administration of uridine itself. Administration of complex II inhibitor 3-nitropropionic acid (3NP) induced neuronal damage in the striatum, substantia nigra and/or thalamus in 80% of the mice and led to 38% mortality. Treatment with PN401 almost completely prevented the neuronal damage due to 3NP and completely prevented mortality. In two subsequent experiments, 3NP-induced weight loss, mortality and behavioral impairment in rotarod performance and spontaneous motor activity were attenuated by treatment with oral PN401. 3NP did not reduce forebrain total uridine nucleotides (TUN), though higher doses of PN401 associated with optimal neuroprotection did elevate TUN to supranormal levels. Thus, oral PN401 treatment has neuroprotective effects in a HD model of mitochondrial dysfunction and the mechanism is more complex than correction of a pyrimidine deficit.

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## 1. Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease characterized by a progressive movement disorder [40]. The polyglutamine repeats in the protein huntingtin lead to symptoms of HD, possibly due to a dysfunction in energy metabolism and consequent vulnerability to excitotoxicity [4,12]. There is a significant deficit in complex II (succinate dehydrogenase; EC1.3.5.1)

enzyme activity found in the striatum postmortem in patients with HD [5,10,12,13,50]. HD has been modeled in experimental rats and mice using 3-nitropropionic acid (3NP), an inhibitor of the complex II enzyme succinate dehydrogenase of the mitochondrial electron transport chain [7,18,27,28,44,45]. Administration of 3NP induces severe motor impairment and loss of selectively vulnerable striatal neurons similar to what is observed in HD patients [2,9].

Neuroprotective effects of the pyrimidine nucleosides uridine and cytidine were first demonstrated more than 40 years ago in an experimental system involving perfusion of circulation-isolated cat brains [21]. Circulating pyrimidines were required to maintain normal electrophysiological ac-

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tivity as well as normal phospholipid and carbohydrate content. Uridine also has cardioprotective effects in the ischemic heart [3,30], improving restoration of ATP following an ischemic episode. The cytoprotective effects of uridine have been proposed to be due to enhanced glycolytic energy production, maintenance of phospholipid and glycogen content. Furthermore, *de novo* synthesis of pyrimidine nucleotides is directly linked to oxidative phosphorylation [31]. An essential enzyme for pyrimidine synthesis dihydroorotate dehydrogenase (DHODH; EC 1.3.99.11) is located on the mitochondrial inner membrane and is functionally coupled to ubiquinone [1]. Complete blockade of the respiratory chain at cytochrome oxidase (EC 1.9.3.1; complex IV) enzyme (though not complex I) activity inhibited DHODH activity in isolated mitochondria, and it was proposed that deficits downstream of ubiquinone in complex III or complex IV mitochondrial respiratory chain enzyme activity could lead to pyrimidine deficits [31].

Uridine itself is poorly absorbed after oral administration, resulting in osmotic diarrhea at doses required to elevate plasma uridine into the anticipated therapeutic range in humans [34]. The limiting factor for therapeutic exploitation of potential neuroprotective effects of pyrimidine nucleosides has been achievement of adequate delivery of uridine. This hurdle has been overcome in the clinic in the context of modulation of the activity of the anticancer agent 5-fluorouracil by the use of triacetyluridine (PN401), an orally bioavailable pro-drug of uridine [26]. Oral administration of PN401 can achieve high micromolar (>100  $\mu$ M) concentrations of circulating uridine in humans [23,26].

These studies addressed whether PN401 protects mice against toxicity associated with mitochondrial dysfunction in the 3NP model of HD.

## 2. Materials and methods

### 2.1. Materials

Phosphodiesterase was obtained from Worthington Biochemical (Lakewood, NJ). *N*-chloroform and methanol of HPLC grade, *n*-butyl acetate, *n*-butanol and hydrochloric acid of reagent grade were obtained from Fisher Scientific (Pittsburgh, PA). Bio-Rad D<sub>C</sub> Protein Assay Kit from Bio-Rad Laboratories (Hercules, CA). All other reagents were obtained from Sigma (St. Louis, MO).

### 2.2. Animals

Male NIH (S) mice (Charles River, Frederick, MD), 7–8 months of age, weighing 30–40 g were housed singly throughout the study. The animals were kept on a 12-h light/dark cycle, with food and water continuously available. The animals were acclimated to our facility for

greater than 7 days before experimentation. Experiments were carried out using procedures that minimized pain and discomfort.

### 2.3. Drug treatments

3NP was made up in sterile water (pH to 7.4 with NaOH) and 3NP was administered to each mouse (i.p.) daily at 4 PM after behavioral testing. The oral dosing of PN401 (Nagase & Co., Chemicals Second Division, 5-1, Nihonbashi-Kobunacho, Chuo-Ku, Tokyo 103, Japan) was 4 g/kg for experiments 1 and 2. The vehicle for PN401 was 0.75% hydroxypropyl-methylcellulose. Oral gavage of PN401 was given b.i.d. with the morning dose given at 9:00 AM and the afternoon dose of PN401 given 1 h prior to 3NP administration. The drug treatments are summarized in Table 1.

PN401 in chow (Harlan-Teklad, Madison, WI) was started 3 days prior to the start of 3NP administration. The mice consumed the same amount of chow (4–5 g/day) in the presence or absence of PN401. A 5% PN401 diet is approximately equal to the 4 g/kg b.i.d. dosing regimen. Oral gavage of PN401 at 4 g/kg or providing PN401 in the chow at a concentration of 6% lead to plasma uridine levels of >50  $\mu$ M (data not shown). The dosing of 3NP in experiments 2 and 3 was reduced from experiment 1 in order to reduce mortality and perform behavioral analysis. Forebrain, heart and liver were quickly frozen at  $-80^{\circ}\text{C}$  until uridine analysis was performed. The same mice that were used in experiment 3 for behavioral analysis were used for uridine and glycogen measurements. Striatum, cortex, heart and liver were obtained at the conclusion of the experiment 3 and frozen at  $-80^{\circ}\text{C}$  until analysis was performed. Mice were sacrificed between 10:00 AM and 3:00 PM.

### 2.4. Behavior

Mice were housed in separate plastic cages in the same room in which the behavioral observation was performed. The behavioral observation was performed between 9:00 AM and 2:00 PM. Spontaneous activity was measured by the Photobeam Activity System (San Diego Instruments, San Diego, CA). Spontaneous activity was quantified by placing mice in the Photobeam Activity System for a period of 30 min. The mice were acclimated once to the activity

Table 1  
Dosing regimen for 3NP and PN401 in Experiments 1–3

Experiment no.	3NP (mg/kg i.p.)	PN401
1 ( $n=8$ /group)	65 d1–4, 50 d5–8	4 g/kg b.i.d. oral
2 ( $n=13$ –14/ group)	40, 50, 60, 60 d1–4, 50 d5–10	4 g/kg b.i.d. oral
3 ( $n=13$ –15/ group)	40, 50, 60, 60 d1–4, 50 d5–12	0, 2, 4, 8% in chow

test and two baseline tests were performed to obtain an averaged baseline activity. The Rotarod apparatus (San Diego Instruments) was used to measure fore- and hindlimb motor coordination and balance. First, mice received rotarod training until they were able to stay on the rotarod at 5, 15 and 25 rpm for the cutoff time of 300, 180 and 180 s, respectively, to be included in the study. The cutoff on the rotarod for experiment 3 was 600 s at 5 rpm. During the training period, each mouse was placed on the rotarod at each speed with a rest period between tests of 2–3 min. The latency to fall off the rotarod within this time period was recorded. Mice received two training runs each day for 4 consecutive days. After training, mice performed two baseline tests that were averaged.

### 2.5. Histology

Animals were anesthetized with pentobarbital (80 mg/kg) and transcardially perfused using a microinjection pump with 10 ml PBS followed by 50 ml of 4% paraformaldehyde. All solutions were ice-cold (4 °C). Brains were removed after fixation, placed in 4% paraformaldehyde solution and (20 brains) shipped to NeuroScience Associates (Knoxville, TN) where all brains were embedded in a gelatin block that was frozen. Sections (40  $\mu$ m) were cut throughout the full length of the brain. The section was stained with cupric silver staining optimized for the detection of degenerating neurons. This method [19,48,49] has been extensively used to identify the extent of neuronal damage. A pathologist (Pathology Associates International, Frederick, MD) who was blinded

to treatment groups in the study analyzed the silver staining.

### 2.6. Measurement of total uridine equivalents in tissues

A modification of a previously described procedure was employed [52]. The mobile phase consisted on 0.15 M potassium dihydrogenphosphate disodium hydrogenphosphate buffer, pH 6.85 (buffer A) and the same buffer containing 20% (v/v) methanol (buffer B). The column was a 5- $\mu$ m Eclipse XDB-C18 reversed-phase column (4.6  $\times$  150 mm; Agilent Technologies, Palo Alto, CA) and a constant flow rate of 1 ml/min was employed. The gradient was: after injection, 7 min in 100% buffer A, during the next 5 min it was changed from 100% to 80% buffer A, during the next 5 min it was changed from 80% to 50% buffer A, these conditions were then maintained for the next 15 min, during the next 1 min conditions were changed from 50% to 100% buffer A, and then the system was maintained during the next 17 min in 100% buffer A before next injection. Samples were homogenized in 2% perchloric acid and the supernatants neutralized with 20% KOH and 1 M Trizma base to pH  $\approx$  9. To convert nucleotides, and nucleotide sugar or lipid derivatives to their constituent nucleosides, 0.5 ml sample was treated with 31 U of alkaline phosphatase and 0.1 U of phosphodiesterase at 37 °C for 1 h. Proteins were denatured by heating for 15 min at 80 °C. Supernatants were diluted 1:4 with running buffer before injection. The identity and separation of compounds was evaluated by their 260/280 absorbance ratio and their retention times compared to

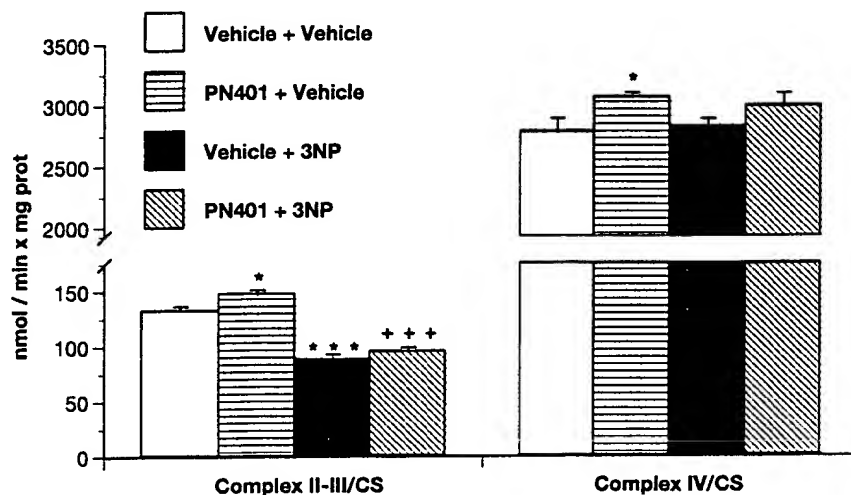


Fig. 1. Mitochondrial respiratory chain enzyme activity measured in forebrain mitochondrial membranes indicates that 3NP reduced complex II–III activity. PN401 did not interfere with inhibition of complex II–III by 3NP. PN401 increased complex II–III and complex IV activities. Symbols \* or \*\*\* indicate  $p < 0.05$  or  $p < 0.001$  significant difference from Vehicle + Vehicle control, respectively. Symbol + indicates  $p < 0.05$  significant difference from Vehicle + 3NP group. Mice were fed for 3 days with a diet of 6% PN401 and forebrains were obtained 3 h after 3NP (65 mg/kg i.p.) injection ( $n = 9–10$ /group). Respiratory chain enzyme activity was corrected for mitochondrial yield by normalizing to the activity of the mitochondrial enzyme citrate synthase (CS) and the data are represented as mean  $\pm$  S.E.M.

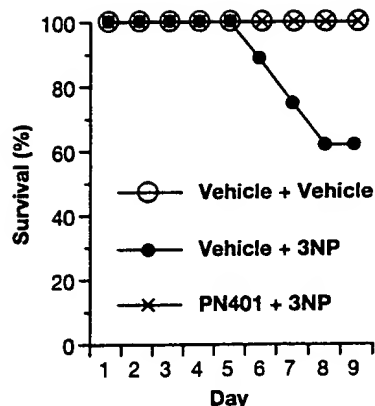


Fig. 2. Oral PN401 (4 g/kg b.i.d.) treatment prevents mortality due to 3NP ( $n=8/\text{group}$ ).

actual standards of nucleotides, nucleotide sugars, nucleosides and bases.

### 2.7. Glycogen assay

A simplified procedure for assay of glycogen based on Parrou and Francois's method [39] was used with some

modification. Briefly, tissue was collected in 0.25 ml of 0.25 M  $\text{Na}_2\text{CO}_3$  and incubated for 4 h at 95 °C. The mixture was brought to pH 5.2 by addition of 0.15 ml of 1 M acetic acid and 0.6 ml of 0.2 M Na-acetate. The mixture (100  $\mu\text{l}$ ) was incubated with 100  $\mu\text{l}$  of *A. niger* amyloglucosidase (final 1.2 U/ml) at RT for 60 min. After incubation, the mixture was centrifuged for 3 min at 3000 rpm. Glucose was determined on 20  $\mu\text{l}$  (adequately diluted in water) of supernatant by addition of 200  $\mu\text{l}$  of glucose oxidase mixture. The mixture was incubated at room temperature for 45 min and absorbance read at 450 nm. Glycogen was calculated as  $\mu\text{mol}$  glucose/g tissue.

### 2.8. Ganglioside assay

The following chemicals and reagents were used for the extraction and analysis of gangliosides and sialic acid according to established procedures with modifications [53]. Extraction of total gangliosides required stirring the tissue homogenate with 10 ml of chloroform (C)/methanol (M) (1:1 by volume) at room temperature for overnight. The supernatant containing total lipids was collected after centrifugation at  $3000 \times g$  for 10 min. The pellet was extracted twice with C/M/ $\text{H}_2\text{O}$  (4:8:3 by volume). All supernatants from each sample were pooled and flash-evaporated. The

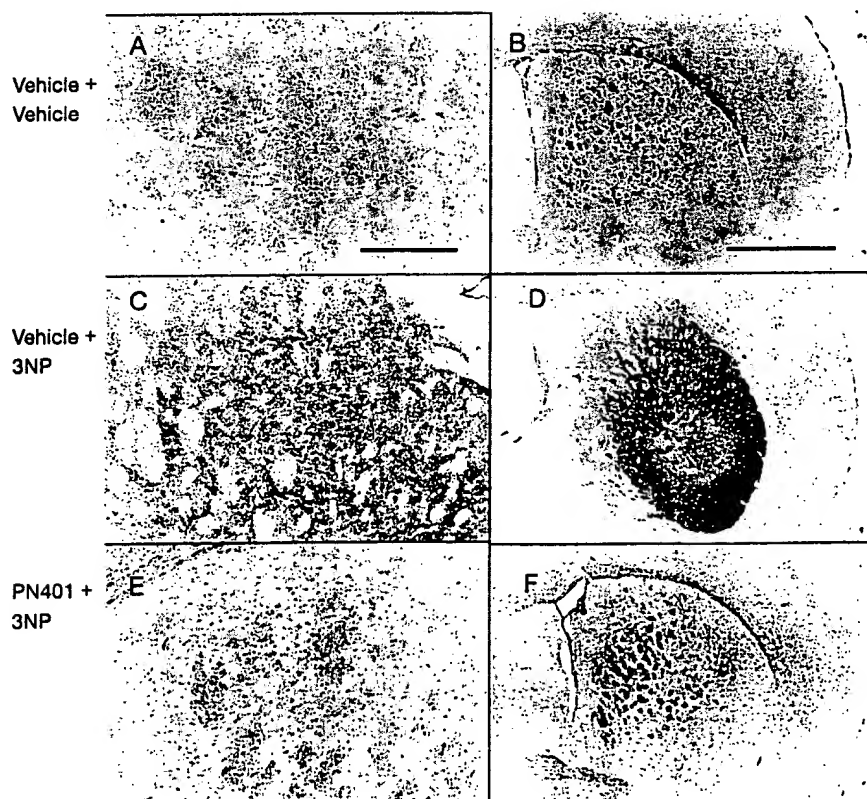


Fig. 3. Oral PN401 treatment protects against striatal neuronal damage due to 3NP. Silver staining of the striatum in mice treated with Vehicle + Vehicle (A, B), Vehicle + 3NP (C, D) or PN401 + 3NP (E, F). The black speckled areas indicate areas of neuronal damage to silver impregnated axons or synaptic terminals. Scale bar in panel A represents 100  $\mu\text{m}$  for panels A, C and E. Scale bar in panel B represents 1 mm for panels B, D and F.

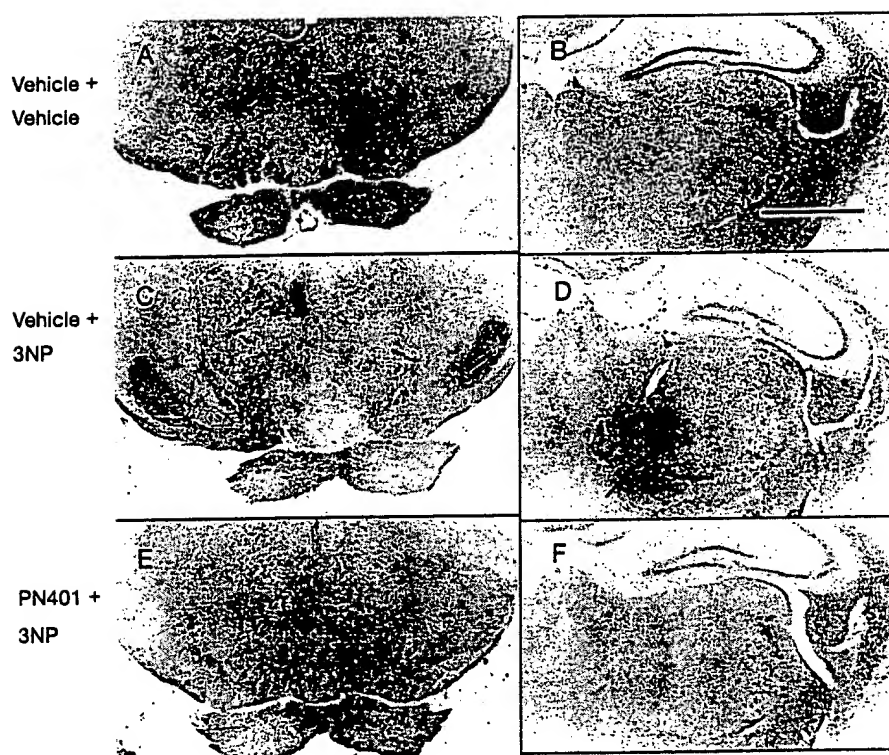


Fig. 4. Oral PN401 treatment protects against neuronal damage in the thalamus and substantia nigra due to 3NP. Silver staining of substantia nigra (A, C, E) and thalamus (B, D, F) in mice treated with Vehicle + Vehicle (A, B), Vehicle + 3NP (C, D) or PN401 + 3NP (E, F). Scale bar in panel B represents 1 mm for panels A–F.

dried lipid extract was dissolved in 1 ml of C/M/H<sub>2</sub>O (30:60:8 by volume, Solvent A) and applied to a DEAE-Sephadex A-25 column (bed volume 1 ml) followed by 1 ml of Solvent A rinse. The neutral lipids fraction was eluted with 5 ml of Solvent A and acidic lipids fraction with equal volume of C/M/1 M ammonium acetate (30:60:8 by volume). The acidic lipid fraction was flash-dried, dissolved in 0.5 ml C/M/H<sub>2</sub>O (4:8:3 by volume) and subsequently applied to Sephadex LH-20 column (bed volume 20 ml) followed by 0.5 ml rinse for desalting. Additional 40 ml of same solvent was added to the column. The first 7 ml eluent was discarded and the subsequent 6 ml of eluent fraction containing the desalted acidic lipids was collected, flash-dried and reconstituted in 1 ml of C/M (1:1 by volume). Gangliosides were separated in the solvent system C/M/0.2% CaCl<sub>2</sub> (50:45:10 by volume) and visualized by fine mist spray of resorcinol–HCl reagent and heating the plate at 95 °C. The appropriate amount of acidic lipid fraction based on 500 mg of protein content was applied to HPTLC plate. A bovine brain ganglioside extract was used as standard. For total sialic acid determination, 300 µl of H<sub>2</sub>O was added to 200 µl of the acidic lipid extract. The mixture was heated at 100 °C for 15 min followed by the addition of 0.5 ml of resorcinol–HCl reagent. The color was extracted into 1 ml of extraction solvent (*n*-butylacetate/*n*-butanol 85:15 by volume) and read at 580 nm using *n*-acetylneuraminic acid as standard.

## 2.9. Respiratory chain enzyme assays

The mice were treated with PN401 (6% diet; Harlan-Teklad) for 3 days. 3NP was administered (65 mg/kg i.p.) and the forebrain was collected 3 h later ( $n=9-10$ /group). The experiment was performed as an acute effect of 3NP on respiratory enzyme activity in order to examine whether PN401 treatment would have any interaction with 3NP. Possible compensatory effects due to chronic administration of 3NP could mask interactive effects of PN401. The objective was to identify the selectivity of 3NP to inhibit

Table 2  
PN401 treatment protects against neuronal damage due to 3NP

Region	Vehicle + Vehicle	Vehicle + 3NP	PN401 + Vehicle
Caudate/putamen	0%	20% (3)	12% (1)
Substantia nigra	0%	40% (1, 3)	0%
Thalamus	0%	80% (1, 2, 2, 2)	0%
Deep mesencephalon	0%	80% (1, 1, 2, 2)	0%
Caudal pontine	0%	60% (1, 1, 3)	0%
Reticular formation	0%	40% (1, 2)	0%

Data are represented as percent mice in each treatment group with apparent damage. The severity of damage was scored 0–3 (scores for each mouse). A score of 0, 1, 2 or 3 corresponded to 0%, 1–25%, 25–50% or 50–75% damage, respectively. The histological analysis was performed on Vehicle+Vehicle ( $n=7$ ), Vehicle+3NP ( $n=5$ ) and PN401+3NP ( $n=7$ ) mice, respectively.

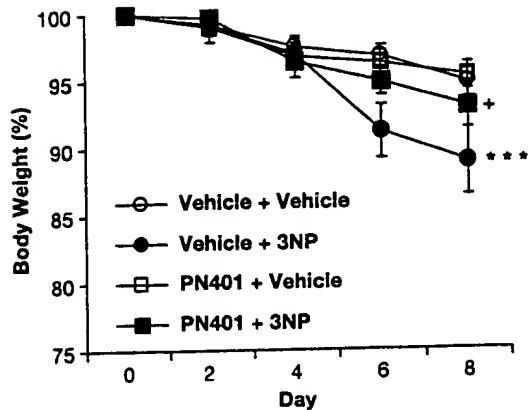


Fig. 5. 3NP induced a decrease in body weight that was attenuated by oral PN401 (4 g/kg b.i.d.). Symbol \*\*\* indicates a significant difference  $p < 0.001$  from Vehicle+Vehicle control. Symbol + indicates  $p < 0.05$  significant difference from Vehicle+3NP group. Data are represented as mean  $\pm$  S.E.M. ( $n = 13-14$ /group).

succinate dehydrogenase activity and to determine if PN401 interfered with the ability of 3NP to inhibit succinate dehydrogenase activity. Complex II–III, complex IV and citrate synthase activities were measured according to the standard procedures [11,55]. Assays were performed on mitochondrial membranes isolated from forebrain by standard subcellular fractionation employing sucrose and Ficoll gradients. The complex II–III and complex IV activities were normalized to mitochondrial citrate synthase activity to account for mitochondrial recovery.

#### 2.10. Statistics

Raw data were analyzed by SigmaStat software (SPSS, Chicago, IL) one-way (group comparison) or two-way

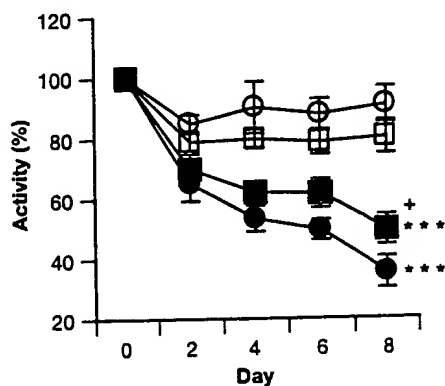


Fig. 6. 3NP induced a decrease in activity that was attenuated by treatment with oral PN401. Symbols for the treatment groups are the same as in Fig. 5 and are as follows: Vehicle+Vehicle (open circles), PN401+Vehicle (open squares), Vehicle+3NP (filled circles) and PN401+3NP (filled squares). Symbol for the statistics are the same as in Fig. 5. Data are represented as mean  $\pm$  S.E.M. ( $n = 13-14$ /group).

(group  $\times$  day comparison) ANOVA followed by comparison of treatments by the Tukey test. Data are represented as mean  $\pm$  S.E.M.

### 3. Results

#### 3.1. Respiratory chain enzyme activity

Mitochondrial respiratory chain enzyme assays were performed to establish the specificity of the effect of 3NP

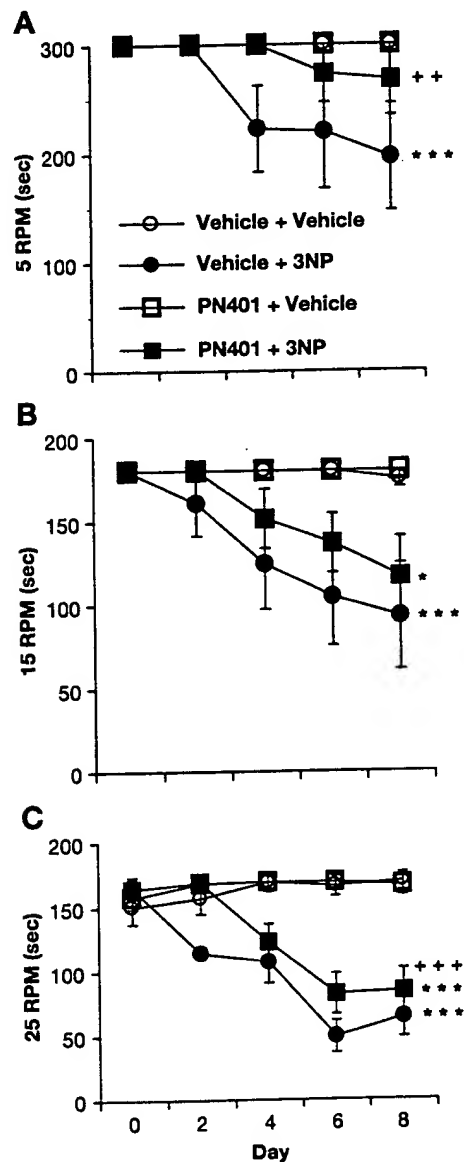


Fig. 7. 3NP induced a decrease in rotarod performance that was attenuated by treatment with oral PN401. Symbols \* or \*\*\* indicate a significant difference of  $p < 0.05$  or  $p < 0.001$  from Vehicle+Vehicle control, respectively. Symbols ++ or +++ indicate  $p < 0.01$  or  $p < 0.001$  significant difference from Vehicle+3NP group, respectively. Data are represented as mean  $\pm$  S.E.M. ( $n = 13-14$ /group).

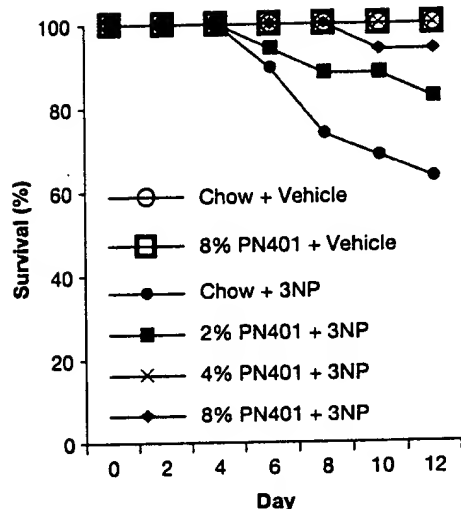


Fig. 8. PN401 in the chow dose-dependently decreased mortality due to 3NP. There was no mortality in the groups Vehicle+Vehicle (open circles), PN401+Vehicle (open squares) or 4% PN401+3NP (closed triangles) groups. The Vehicle+3NP (closed circles) had the poorest survival followed by 2% PN401+3NP (closed squares) and 8% PN401+3NP (closed diamonds). Data are represented as mean  $\pm$  S.E.M. ( $n=13-16$ /group).

in our system and any possible interaction between 3NP and PN401. 3NP specifically reduced complex II–III activity by 33% ( $p<0.001$ ), but had no effect on complex IV activity (Fig. 1). In the presence of PN401, 3NP inhibited complex II–III activity by 36%. Therefore, PN401 did not interfere with the ability of 3NP to inhibit complex II–III activity. However, PN401 treatment led to an 11% and 8% increase in complex II–III activity in the absence and presence of 3NP ( $p<0.05$ ). Furthermore, PN401 treatment increased complex IV activity by 11% ( $p<0.05$ ) and 6% in the absence and presence of 3NP.

### 3.2. Experiment 1: the effect of PN401 on mortality and neurodegeneration due to 3NP

PN401 completely prevented mortality due to 3NP treatment (Fig. 2). There was pronounced silver staining of axons and synaptic terminals in the striatal area (caudate/putamen area), substantia nigra and thalamus in the control+3NP treated mice (Fig. 3). Silver impregnation of axons and/or synaptic terminals in the thalamus, deep mesencephalon and/or reticular formation (caudal pontine area) was also found in 80% of the Vehicle+3NP treated mice (Fig. 4; Table 2). The thalamus, substantia nigra and striatum were especially vulnerable to damage by 3NP. The damage due to 3NP was almost completely prevented by PN401.

### 3.3. Experiment 2: the effect of PN401 on behavioral impairment due to 3NP

In experiment 2, oral gavage of PN401 decreased mortality due to 3NP. There was 20% (3/15) mortality

due to 3NP versus 7% (1/15) mortality in the PN401+3NP treatment group. There was a loss of body weight due to 3NP treatment that was significantly ( $p<0.05$ ) attenuated by PN401 treatment (Fig. 5). PN401 treatment alone had no effect on body weight. There was a decrease in body weight in both the Vehicle+Vehicle and PN401+Vehicle groups that may be attributed to oral gavage. Oral gavage of a large volume of liquid fills up the stomach and may consequently decrease feeding. 3NP treatment significantly ( $p<0.001$ ) decreased activity of mice and this decrease was significantly ( $p<0.05$ ) attenuated by treatment with PN401 (Fig. 6). PN401 treatment alone did not affect activity level compared to the Vehicle+Vehicle treatment control group. Rotarod performance was significantly impaired at 5, 15 and 25 rpm due to 3NP treatment (Fig. 7). PN401 treatment attenuated impairment due to 3NP treatment at 5 rpm ( $p<0.01$ ) and 25 rpm ( $p<0.001$ ). PN401 treatment alone did not affect rotarod performance at 5, 15 or 25 rpm compared to the Vehicle+Vehicle treatment control group.

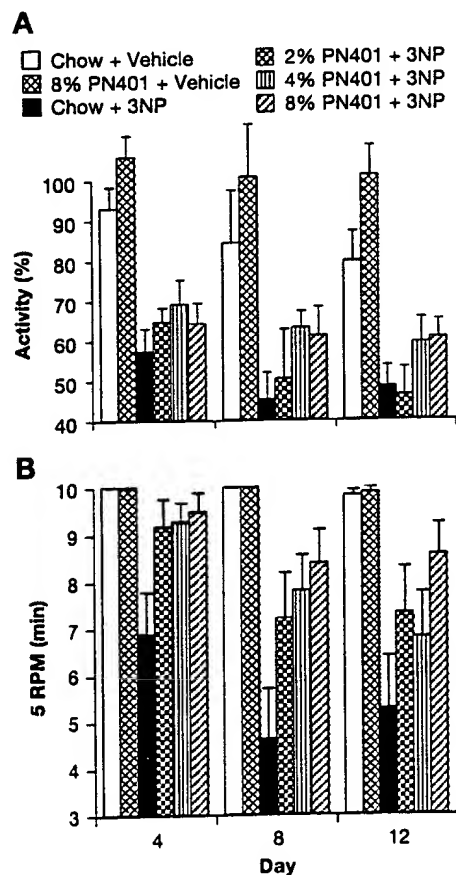


Fig. 9. PN401 attenuates the hypoactivity and rotarod impairment induced by 3NP. (A) The higher doses of PN401 of 4% and 8% were required to attenuate hypoactivity ( $p<0.05$ ), whereas (B) all doses of PN401 improved performance on the rotarod ( $p<0.01$ ). Data are represented as mean  $\pm$  S.E.M. ( $n=13-16$ /group).

### 3.4. Experiment 3: dose–response effects of PN401 on toxicity due to 3NP

PN401 decreased mortality in a dose-dependent manner due to 3 NP with 4% and 8% PN401 in the chow protecting against mortality better than 2% PN401 (Fig. 8). The decrease in body weight due to 3NP was modest in this experiment. PN401 attenuated the decrease in body weight, but this effect did not reach significance. PN401 attenuated the hypoactivity due to 3NP at 4% and 8%, but not at 2% PN401 in the chow (Fig. 9). PN401 also decreased motor impairment due to 3NP at 2% ( $p < 0.05$ ) as well as 4% and 8% ( $p < 0.001$ ) PN401 in the chow (Fig. 9). One potential effect of pyrimidines to enhance energy reserve would be an increase in liver glycogen production since uridine diphosphate-glucose is a cofactor for glycogen synthesis. Liver glycogen levels were significantly ( $p < 0.001$ ; two-way ANOVA for a treatment effect) reduced by 40% due to 3NP in the presence or absence of PN401 (Fig. 10A). However, PN401 treatment did not affect liver glycogen concentration in the presence or absence of 3NP. Another possible effect of PN401 could be an increase in ganglio-

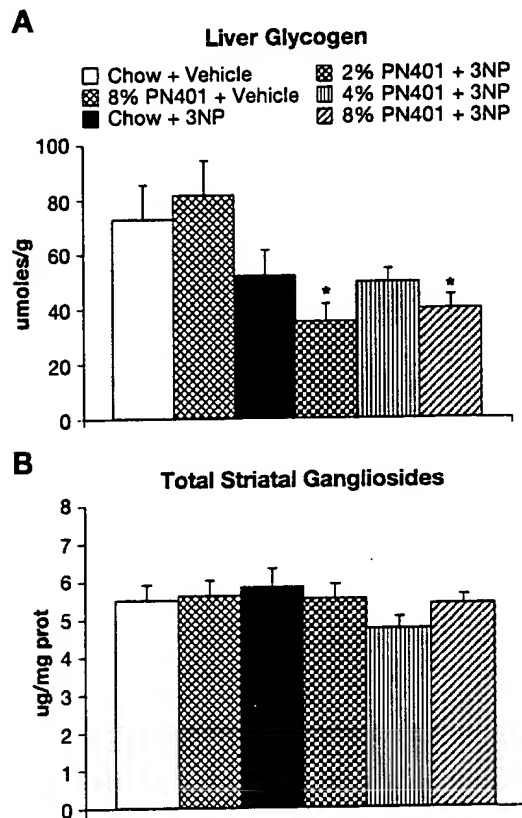


Fig. 10. PN401 did not have an effect on (A) liver glycogen or (B) striatal gangliosides. There was a significant ( $p < 0.001$ ) decrease in liver glycogen due to treatment with 3NP regardless of the presence or absence of treatment with PN401. Symbol \* indicates a significant difference of  $p < 0.05$  from Vehicle+Vehicle control. Data are represented as mean  $\pm$  S.E.M. ( $n = 13$ –16/group).

Table 3

The effect of PN401 and 3NP treatment on the total uridine nucleotide pool

Treatment	Forebrain	Heart	Liver
Vehicle + Vehicle	303.0 $\pm$ 8.6	234.9 $\pm$ 5.3	1528.2 $\pm$ 20.3
8% PN401 + Vehicle	330.0 $\pm$ 9.4	309.6 $\pm$ 39.1	2029.4 $\pm$ 79.7***
Vehicle + 3NP	291.9 $\pm$ 9.3	240.9 $\pm$ 11.8	1496.1 $\pm$ 48.8
2% PN401 + 3NP	301.2 $\pm$ 5.9	262.8 $\pm$ 18.8	1595.7 $\pm$ 32.1
4% PN401 + 3NP	313.1 $\pm$ 8.5	302.2 $\pm$ 21.8	1538.0 $\pm$ 28.0
8% PN401 + 3NP	330.9 $\pm$ 8.7--	417.7 $\pm$ 29.6---	1984.4 $\pm$ 96.7---

TUN was measured as nmol uridine/g tissue.

\*\*\* Indicates  $p < 0.001$  compared to Vehicle + Vehicle.

-- Indicates  $p < 0.01$  compared to Vehicle + 3NP.

--- Indicates  $p < 0.001$  compared to Vehicle + 3NP.

sides that buffer calcium [15] and have neuroprotective activity [22]. Total striatal ganglioside content was unchanged by PN401 and/or 3NP administration (Fig. 10B). An analysis of the specific pattern ganglioside (GM1, GD1a, GD1b and GT1b) content of selected samples indicated that there was no change in the pattern of ganglioside content in the different treatment groups (data not shown).

The total uridine nucleotide (TUN) pool was measured in the forebrain, heart and liver to evaluate whether treatment with 3NP and/or PN401 would affect the total pool of uridine and its derivatives including phosphorylated and glycosylated forms. The liver has been shown to be very active in de novo pyrimidine synthesis [54] and therefore it was not surprising that the TUN content in descending concentration was liver  $\gg$  forebrain  $\gg$  heart (Table 3). 3NP had no effect on the TUN pool in the forebrain, heart or liver. Treatment with 8% PN401 alone led to an increase of 11%, 13% and 33% in TUN content in the forebrain, liver and heart, respectively. In a similar manner, PN401 dose-dependently increased the TUN pool in the forebrain, liver and heart in the presence of 3NP. The increase in TUN pool in the heart was most dramatic, especially in the presence of 3NP. The total adenosine nucleotide pool was also measured and there was no change due to 3NP and/or PN401 treatment (data not shown).

## 4. Discussion

Oral administration of PN401 demonstrated neuroprotective effects in the 3NP model of HD. PN401 enables oral delivery of the high levels of uridine that were required for maximal neuroprotective activity. The primary features of HD in this model, including mortality, motor impairment, weight loss and neuronal degeneration, were all decreased by PN401 treatment in these studies. The delivery of high levels of uridine may affect the neurodegenerative process by numerous pathways, given that uridine has a broad range of metabolic functions.



PN401 consistently decreased mortality due to 3NP in these experiments. Likewise, PN401 attenuated the loss in body weight due to 3NP. The cause of the 3NP-induced mortality and loss of body weight may be partially due to factors outside the CNS [20]. Patients with late stage HD exhibit dysphagia and loss of body weight [40]. However, mild weight loss in patients with early stage HD is typically associated with increased energy utilization rather than dysphagia.

The silver staining indicates neuronal damage to several brain regions. Systemically administered 3NP has previously been shown to damage the striatum, substantia nigra and thalamus [36]. In addition, 3NP treatment resulted in damage to the reticular formation, deep mesencephalon and caudal pontine area. PN401 nearly completely prevented neuronal damage in all of these regions.

Motor impairment induced by 3NP was evident in hypoactivity and inability to stay on the rotarod. Typically, the course of HD begins with involuntary movements (chorea), then a loss of control of voluntary movements that eventually progresses to rigidity and dystonia. The later stages of HD that present as poor motor coordination and hypoactivity are more closely modeled by 3NP administration. PN401 reversed the effects of 3NP on activity and rotarod performance. Treatment with PN401 alone to normal animals did not affect activity level or rotarod performance.

It has already been established that oral administration of PN401 is well tolerated in humans and can elevate plasma uridine to concentrations greater than 100  $\mu\text{M}$  [26]. The acetate substituents on PN401 prevent the uridine moiety from being degraded by the catabolic enzyme uridine phosphorylase, which is present in very high concentrations in the intestine. Furthermore, PN401 is efficiently absorbed into the circulation after oral administration and is then rapidly deacetylated by nonspecific esterases yielding free uridine. Uridine is readily taken into the brain from the circulation by a specific pyrimidine nucleoside transporter [14].

Does impairment of mitochondrial function lead to a deficiency in pyrimidines *in vivo*? Furthermore, does the neuroprotective effect of PN401 require the correction of a pyrimidine deficiency? There was no effect of the complex II inhibitor 3NP on the total uridine nucleotide (TUN) pool in various tissues sampled from the mice in the present study. This finding is consistent with the suggestion of Löffler et al. [31] that inhibition of the mitochondrial respiratory chain upstream of complex III would not affect *de novo* synthesis of pyrimidines. However, treatment with the complex IV inhibitor sodium azide administered continuously via subcutaneous osmotic minipump at a concentration that led to significant mortality did not decrease TUN in the brain (data not shown). Therefore, degrees of complex IV respiratory chain inhibition sufficient to have lethal consequences *in vivo* did not impair *de novo* biosynthesis of pyrimidines. Despite a lack of evidence for measurable deficits in pyrimidine nucleotide pools due to 3NP, exogenous uridine provided by oral administration of PN401 yielded significant

neuroprotective effects. Administration of PN401 resulted in a dose-dependent increase in the TUN pool in the forebrain, heart and liver. This increase in the TUN pool was similar in the presence or absence of 3NP. Thus, the neuroprotective effects were not due to correction of a putative deficit caused by interference of the mitochondrial respiratory chain, but may represent a pharmacological improvement of energy metabolism or prevention of cell dysfunction or death due to excitotoxicity or calcium flooding.

In a single-dose experiment, 3NP decreased the activity of complex II–III, but not complex IV, 3 h after administration. Treatment with PN401 in the chow for 3 days did not interfere with the ability of 3NP to decrease complex II–III activity. The protective effects of PN401 are evidently not due to a prevention of the mitochondrial respiratory chain inhibition by 3NP. It was notable that treatment with PN401 led to a modest significant increase in complex II–III and complex IV activities in both the absence and presence of 3NP. Therefore, improved respiratory chain function may contribute to the neuroprotective effects of PN401.

Is there a link between respiratory chain dysfunction and a requirement for pyrimidines? Dependence upon high micromolar levels of exogenous uridine has been demonstrated in cells with mtDNA mutations as well as normal cells subjected to antiretroviral dideoxynucleosides which damage mitochondrial DNA [25], chloramphenicol which inhibits mitochondrial protein synthesis [37], ethidium bromide which depletes mitochondrial DNA [16,29] and anaerobic conditions [51]. There is extensive support for the role of mitochondrial dysfunction in the etiology of neurodegenerative disorders [4,6,8,35,38,42]. Cybrid cell lines have been used to demonstrate that mitochondrial DNA from patients with Parkinson's [46] and Alzheimer's disease [47] can induce mitochondrial dysfunction in cells with normal nuclear DNA (HD is a result of a nuclear DNA CAG repeat expansion). These recipient cells, termed Rho 0 cells, with their mitochondrial DNA depleted by treatment with ethidium bromide, require high micromolar uridine and pyruvate for survival.

Given that respiratory chain dysfunction can lead to a requirement for uridine, what is the level of uridine required for neuroprotective activity? Since 3NP treatment did not induce a pyrimidine deficit, the level of uridine required must be above normal plasma levels (2–10  $\mu\text{M}$ ) [41]. We have observed that 6% PN401 in the chow increases plasma uridine levels above 50  $\mu\text{M}$ . Therefore, the result indicating that 4% and 8% PN401 in the chow was more protective compared to 2% PN401 in the chow suggests that high ( $\geq 30$   $\mu\text{M}$ ) plasma levels of uridine must be achieved for a maximal therapeutic effect. In contrast, much lower concentrations (1–5  $\mu\text{M}$ ) of uridine are sufficient to compensate for complete blockade of *de novo* pyrimidine synthesis with the carbamoyl phosphate synthetase inhibitor PALA [24]. This again suggests that therapeutic effects of PN401 in the 3-NP model are not simply a consequence of correction of possible deficits in pyrimidine synthesis secondary to respiratory chain inhibition.

What is the mechanism of neuroprotective activity for these high levels of uridine? There will likely not be a single mechanism of action for uridine given that uridine has such diverse metabolic functions. Uridine availability is important for RNA, DNA and phospholipid synthesis. Uridine, in the form of UDP sugars, is a cofactor in the enzymatic glycosylation reactions in the synthesis of glycogen, glycoproteins and glycolipids.

The glycolipid GM1 has been shown to have neuroprotective activity in models of Parkinson's [43] and HD [32] as well as buffering intracellular calcium [15]. Total striatal gangliosides or specific gangliosides such as GM1 were not changed by PN401 treatment alone or in combination with 3NP. However, the lack of changes in liver glycogen and striatal gangliosides may be due to the 12–20 h of time between sacrifice and the last dose of 3NP or PN401. Therefore, the potential role of glycogen or gangliosides in the mechanism of neuroprotective activity of PN401 cannot be ruled out.

In isolated perfused hearts, uridine improves the efficiency of glycolytic ATP production [17] and accelerates restoration of ATP content during recovery from ischemia [33]. Geiger and Yamasaki [21] reported that uridine and cytidine helped to maintain cerebral energy metabolism during acute failure in cats with isolated cerebral circulation. These authors found that cytidine and uridine added to the cerebral perfusate increased brain glucose uptake and simultaneously reduced lactate accumulation. Increased glucose utilization without lactate buildup implies that oxidative phosphorylation may have been augmented by the pyrimidines, though the mechanism is unknown. Further studies are required to confirm and elucidate whether PN401 is improving metabolic efficiency at the level of respiratory chain enzyme activity and what specific metabolic intermediates such as phospholipids are affected by PN401.

Supraphysiological concentrations of uridine appear to have significant neuroprotective effects even when there is not a measurable pyrimidine deficiency underlying the neuropathology. PN401 has also been shown to increase lifespan in the N171-82Q transgenic model of HD (study in progress). Treatment with PN401 ameliorated the mortality, motor impairment, weight loss and neuronal degeneration in the 3NP mitochondrial toxin model of HD. The ability of PN401 to protect against neurodegeneration due to mitochondrial dysfunction may suggest an important therapeutic potential for PN401 in the treatment of HD and possibly other neurometabolic disorders.

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